Human CYP2A6 activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK): mutational specificity in the *gpt* gene of AS52 cells

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The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potential human carcinogen that is known to be metabolized to DNA-reactive intermediates by the cytochromes P450. We have examined th ıture of NNK's DNA damaging effects in a mammalian cell system expressing a specific human cytochrome P450 (2A6) and containing a target gene for mutagenesis. Human CYP2A6, which is known to activate NNK to a mutagen, was lipofected via a retroviral vector into the Chinese hamster ovary AS52 cell line, which contains the bacterial *gpt* gene and can be mutated to 6-thioguanine resistance. AS52 cells expressed negligible CYP2A6-specific coumarin 7-hydroxylase activity (0.7 pmol/mg protein/min), while a CYP2A6 transfected clone (AS52-E8) expressed 30 pmol/mg protein/min. Both cell lines were equally sensitive to the cytotoxic and mutagenic effects of the direct-acting mutagen ethylmethanesulfonate; however, only the AS52-E8 cells exhibited a dose-dependent increase in cytotoxicity and mutant frequency upon treatment with NNK. At the highest NNK dose (1200 μg/ml), the mutant frequency in AS52-E8 cells was 14-fold (339×10^{-6}) greater than the spontaneous frequency of 24×10^{-6}. Ninty-eight mutant clones were isolated following NNK treatment. Based on PCR analysis, 21 clones contained deletions/rearrangements and 77 were putative point mutants. Sequencing po tial point mutants showed that 81% contained G:C to A:T transitions. Four of six G:C to A:T hotspots were at the second G of the GGT motif, which is the motif and major mutation found in codon 12 of Ki-*ras* from NNK-induced lung tumors in strain A mice. Since NNK may be metabolized via different pathways to pyridyloxobutylate or methylate DNA, the data suggest that methylation damage causes the major mutagenic events in AS52-E8 cells when NNK is activated by human CYP2A6.

[1]**Abbreviations:** NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; EMS, ethylmethanesulfonate; HBSS, Hank's balanced salt solution; PCR, polymerase chain reaction; S-9, 9000 *g* post mitochondrial supernatant; CYP, cytochrome P450; NDMA, *N*-nitrosodimethylamine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 6TG, 6-thioguanine; 6TG^r, 6-thioguanine resistant; *dhfr*, dihydrofolate reductase; *gpt*, guanine phosphoribosyltransferase.

Introduction

The tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) is a potent carcinogen in rodents (1,2) and is believed to play a role in human carcinogenesis (1,3,4). Metabolic activation of NNK is required for manifestation of its carcinogenic activity. NNK may be metabolized via several pathways, with α-hydroxylation at the methylene or methyl carbons adjacent to the *N*-nitroso group leading to DNA methylation or pyridyloxobutylation, which are thought to be the pathways responsible for initiation of carcinogenesis (5–8). Pyridine *N*-oxidation is also known to occur and is believed to be a detoxification pathway (9,10). Carbonyl reduction to the alcohol is a major metabolic pathway (5,11) whose products can either be conjugated (12,13) or undergo α-hydroxylations leading to DNA-reactive intermediates (14,15). Several of the cytochromes P450 have been implicated in the various metabolic pathways for NNK in mice, rats and humans (16–20); however, the extent to which individual P450s metabolize NNK and their contribution to NNK's species-dependent organotrophic effects remains unclear.

NNK has been shown to be mutagenic both *in vivo* and *in vitro*, and in some systems the nature of the mutagenic lesions has been elucidated. In rodents, NNK has been shown to induce tumors in several tissues including liver, lung and nasal cavity, although the type of mutation in suspected relevant genes (e.g. *ras*) can vary with species, strain and tissue. To date, gene-specific mutagenesis studies with NNK have been conducted primarily in lung tissues. NNK has been shown to induce lung tumors which contain a high percentage of GC to AT transitions in codon 12 of the Ki-*ras* gene in susceptible strain A mice (21,22). Similar results have been reported for hamster lung tumors induced by NNK (23). However, in resistant mouse strains and in the rat, *ras* mutations in lung tumors are less frequent or not detected (24,25). Furthermore, examination of strain A mouse lung tumors induced by NNK, or by synthetic precursors simulating either NNK methyl hydroxylation (NNKOAc) or methylene hydroxylation (AMMN), has shown that NNK produced mostly O^6-methylguanine-like mutations in Ki-*ras*. AMMN produced only this type of mutation, while NNKOAc was shown to induce a more complex mutational pattern (22). Thus, it is possible that differences in the P450 isozymes, and the sites at which α-hydroxylation occurs, may account in part for the species/tissue differences in *ras* activation. *In vitro*, NNK has been shown to be mutagenic in human lymphoblastoid cells expressing transfected cytochromes P450, with the relative orders of activation being CYP 2A6 = 1A2 > 2E1 > 2D6 (17). In human liver microsomes, the cytochromes P450 2A6, 1A2 and 2E1 are reported to be the major activators of NNK to intermediates that caused DNA damage (18,20).

These data suggest that different pathways for NNK activation exist, and that they influence the nature of NNK's mutagenic effects. Thus, gaining knowledge of which cytochromes P450 produce methylating and/or pyridyloxobu-

tylating intermediates, as well as the nature of the resulting DNA damage, may help in elucidating NNK's species- and tissue-specific carcinogenic and mutagenic effects. In previous studies we employed approaches whereby transfected individual cytochromes P450 were stably expressed in the mammalian cells within which the biological effects of the xenobiotic were measured (26,27). We have reported that stably expressed human cytochrome P450 2A6 activated NNK to mutagenic and oncogenic intermediates (17,28,29). Our choice of CYP2A6 has been based on observations that members of the P450 2A family in mice, rats and humans have been implicated in the metabolism of nitrosamines, including NNK (16,18–20) and that human CYP2A6, along with 2E1 and 1A2, has been indicated as a major activating enzyme in human liver (20). In the present study we have utilized Chinese hamster ovary derived AS52 cells carrying the bacterial *gpt* gene (30) to investigate the nature of the mutations induced in this gene when NNK is activated by stably expressed human CYP2A6.

Materials and methods

Materials

NNK was obtained from Chemsyn Science Laboratories (Lenexa, KS). Ethyl methanesulfonate (EMS) was from Eastman Chemical Company (Rochester, NY). Oligonucleotide primers for PCR and sequencing were synthesized by Research Genetics (Huntsville, AL). Taq polymerase was purchased from Perkin-Elmer (Norwalk, CT). Sequenase (version 2.0) was purchased from US Biochemical Corp. (Cleavland, OH). [γ-^{33}P]dATP was obtained from NEN/Du Pont (Wilmington, DE).

Construction of the vector

The construction of the pMV7 retroviral vector carrying a copy of the CYP2A6 cDNA has been described (28). The pMV7 vector had been kindly supplied by Dr. L.Bernard Weinstein (Columbia University, NY).

Coumarin 7-hydroxylase assay

The whole cell and microsomal assays were modified from the procedure of Greenlee and Poland (31). Microsomal assays were performed as previously reported (28). For the whole cell assay, cells were incubated overnight with media containing 10 μM coumarin. After 16 h, an aliquot of the media was centrifuged to remove debris and 0.5 ml was then added to 2.5 ml of 0.25 M Tris–glycine buffer (pH 9.0). Fluorescence was measured in a Farrand spectrofluorometer (λex = 368, λem = 456) against a time zero blank consisting of media containing 10 μM coumarin. Coumarin 7-hydroxylase activity measured in the whole cell assay correlates linearly with that from the microsomal assay (M.Hosokawa, unpublished data).

Cell culture conditions

All cells were grown in tissue culture incubators at 37°C in 5% CO_2 and 98% relative humidity. The ψ-2 packaging cell line (32) generously supplied by Dr R.Mulligan (Whitehead Institute for Biomedical Research, Cambridge, MA) was maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 5% bovine calf serum (Hyclone, Logan, UT), penicillin–streptomycin and L-glutamine as previously reported (28). The PA317 packaging cell line (33), obtained from the American Type Culture Collection (Rockville, MD), was maintained in DMEM supplemented as for ψ-2 cells. AS52 culture conditions have been previously described (30). The media for AS52 culture and selection have been defined as follows: F12FCM5 is Ham's F12 with 5% dialyzed, heat-inactivated fetal bovine serum; MPA medium is F12FCM5 plus 250 μg/ml xanthine, 25 μg/ml adenine, 50 μM thymidine, 3 μM aminopterin and 10 μg/ml mycophenolic acid; and 6-TG medium is F12FCM5 (hypoxanthine-free) plus 10 μM 6-TG.

Infection and lipofection of cells and isolation of expressing clones

Amphotropically packaged pseudoretroviral particles carrying CYP2A6 were produced in the PA317 cell line. PA317 packaging cells were infected by incubation in medium plus 10 μg/ml polybrene with ecotropically packaged particles from a ψ-2 producer cell line (28). After selection in DMEM containing 440 μg/ml G418, infected PA317 cells were subcultured to obtain virus-producing cultures. The packaging cells were cultured in DMEM containing 1% serum for 16 h and the virus containing media were collected. Because of the difficulty of infecting Chinese hamster cells with murine retroviruses, we utilized a procedure we had reported earlier (34) for transferring the amphotropically packaged vector containing CYP2A6 into AS52 cells. AS52 cells were incubated for 16 h in F12 medium (1% serum) plus an equal volume of DMEM (1% serum) containing the packaged virions (4×10^5 c.f.u./ml when titered on AKR cells) plus 16 μg/ml Lipofectin (Life Technologies, Gaithersburg, MD). The cells were then maintained on F12FCM5 for 3 days and F12FCM5 with 400 μg/ml G418 thereafter until colonies were isolated at 12 days. Individual colonies were subcloned into 35 mm dishes in F12FCM5. After ~5 days the cloned cells were split and one dish of each clone was checked for coumarin 7-hydroxylase activity using a whole cell assay. Three clones with high activity were each diluted to 1 cell/ml and again subcloned at 0.1ml/well in a 96 well tissue culture cluster. After 2 weeks, cells were transferred to individual 60 mm dishes. Each subclone was checked for coumarin 7-hydroxylase activity and stocks were frozen. One clone, AS52-E8, was used in all subsequent studies.

Mutation of AS52 and isolation of mutants

Stock AS52 cells and the AS52-E8 clone were routinely maintained in MPA medium. For the isolation of mutants, 1 day prior to chemical treatment, 1×10^6 cells were replated into each of 29 100 mm dishes in F12FCM5 plus xanthine, adenine and thymidine at 0.25× the concentration used in MPA medium. On the day of treatment, each dish was washed with HBSS and 10 ml of F12 containing 1200 μg/ml NNK (25 dishes) or 300 μg/ml EMS (two dishes) was added. Twenty-four hours following the end of the treatment, the cells were subcultured at 10^6 cells/100 mm dish. At this time, three 60 mm dishes were seeded at 200 cells/dish with cells from the control and EMS treatment dishes, and from 5/25 NNK treatment dishes, to measure survival. Cells from all 25 NNK treatment dishes were subcultured twice more at 10^6 cells/100 mm dish at 48 h intervals and finally on day 6 were plated in duplicate at 2×10^5 cells/100 mm dish for selection of mutants in 6-TG medium. Again, at this time survival was determined as described above so that a cloning efficiency could be determined and used to calculate the respective mutation frequencies for control, EMS- and NNK-treated cells. After 8 days in selection, three well-separated colonies were isolated from each NNK-treated dish and placed in individual wells of a 12 well tissue culture cluster. Records of the treatment dish of origin were maintained for each clone collected. Studies to determine EMS and NNK dose response were done with similar protocols except that two dishes were initially seeded per EMS or NNK dose.

DNA isolation

DNA for PCR amplification was isolated from 2.5×10^7–1×10^8 trypsin-released cells of each mutant clone with an Oncor Non-organic DNA Extraction Kit (Oncor, Gaithersburg, MD) following the directions supplied by the manufacturer.

PCR amplification and sequencing

Amplification of the *gpt* gene was carried out as previously described (30,35). Briefly, in the first PCR, AS52 wild-type or mutant genomic DNA was amplified using primers specific for the *gpt* gene and also primers for exon 5 of the *dhfr* gene. The latter provided an internal positive control for each reaction. The products of the primary PCR were electrophoresed through a 1% low melting point agarose gel. Up to three bands were observed on the gel after this PCR (35). One band, migrating at 1.1 kb, is the PCR product of a rearranged pSV*gpt* insert that does not contain a functional *gpt* gene but is in proximity to the functional *gpt* gene. The 0.7 kb band is the *gpt* structural gene PCR product. The band at 0.4 kb is the PCR product of exon 5 of the *dhfr* gene and is the positive control for the PCR reaction. The 0.7 kb band corresponding to the full-length *gpt* gene was isolated and used as the template for the second PCR. This second PCR reaction utilized a 5′-primer internal to that used in the primary PCR with the same 3′-primer and produced a full-length *gpt* product suitable for DNA sequencing. Sequencing was carried out essentially as described (30) with the exception that the primers were end-labeled with [γ-^{33}P]ATP (NEN/Du Pont). G to A transitions at positions 115 and 116 resulted in anomalous mobility of the DNA at these lengths and were resolved by running the sequencing products on gels containing 40% formamide.

Results

Selection and characterization of 2A6 expressing clones

Thirty-seven G418-resistant clones were isolated from two Lipofectin-mediated infections of AS52 with PA317-packaged retroviral particles containing the pMV7/2A6 vector. Whole cell coumarin 7-hydroxylase activities for 22 of the lines established from these clones ranged from 120 to 1810 nmol/10^6 cells/16 h. The parental cell line, AS52, averaged 3.9 nmol/10^6 cells/16 h. Three of the clones with the highest coumarin 7-hydroxylase activities, and which exhibited good

morphologies and growth rates, were recloned by dilution cloning. After reseeding and expansion, a subclone with coumarin 7-hydroxylase activity (1955 nmol/10^6 cells/16 h) approximately the same as the initial clone was chosen and was designated AS52-E8. This clone's activity was measured over a 3 month period of continuous culture and found to remain constant in the absence of G418. Clone AS52-E8 was used for all subsequent studies.

Characterization of the AS52-E8 cell line

The clone AS52-E8 had a growth rate and morphological appearance similar to wild-type AS52 cells. By Southern analysis, clone AS52-E8 was found to contain one copy of integrated CYP2A6 cDNA (data not shown). This clone expresses a microsomal coumarin 7-hydroxylase activity of 30 ± 5 pmol/mg microsomal protein/min compared to a background level of 0.7 ± 0.2 pmol/mg microsomal protein/min in wild-type AS52. This activity is within the range of that previously reported for human liver tissue samples (20.36).To determine whether vector integration or CYP2A6 expression affected the cloning efficiencies, cytotoxic sensitivity or mutational response of AS52-E8 compared to the parental AS52 cells, the clones were treated with the direct-acting mutagen EMS. Both cell lines exhibited similar cloning efficiencies and were equally sensitive to the cytotoxic and mutational effects of a 5 h treatment with EMS (Figure 1). EMS at concentrations up to 600 µg/ml produces relatively little cytotoxicity in both the parental and the E8 clone (Figure 1a). The induced mutant frequency by EMS in both lines is nearly identical at all doses and shows an essentially linear dose response up to 600 µg/ml at which point both induced mutant frequencies were 735×10^{-6} or ~23-fold over the spontaneous mutant frequency (Figure 1b).

To determine whether CYP2A6 expression increased the sensitivity of the cells to a chemical that this enzyme is known to activate, the parental and AS52-E8 clones were treated with NNK for 24 h. NNK was not cytotoxic to the non-metabolizing parental AS52 cells at concentrations up to 1200 µg/ml, and only the CYP2A6-expressing AS52-E8 cells showed a dose-dependent increase in cytotoxicity and mutant frequency upon treatment with NNK (Figure 2). At the highest NNK dose assayed (1200 µg/ml), the induced mutant frequency in AS52-E8 cells was 12-fold (405×10^{-6}) over a spontaneous mutant frequency of 32×10^{-6}. These results indicate that CYP2A6 expression provides AS52 cells with the ability to metabolize NNK, and that the expressing cells exhibit a dose response to the cytotoxic and mutagenic effects of NNK.

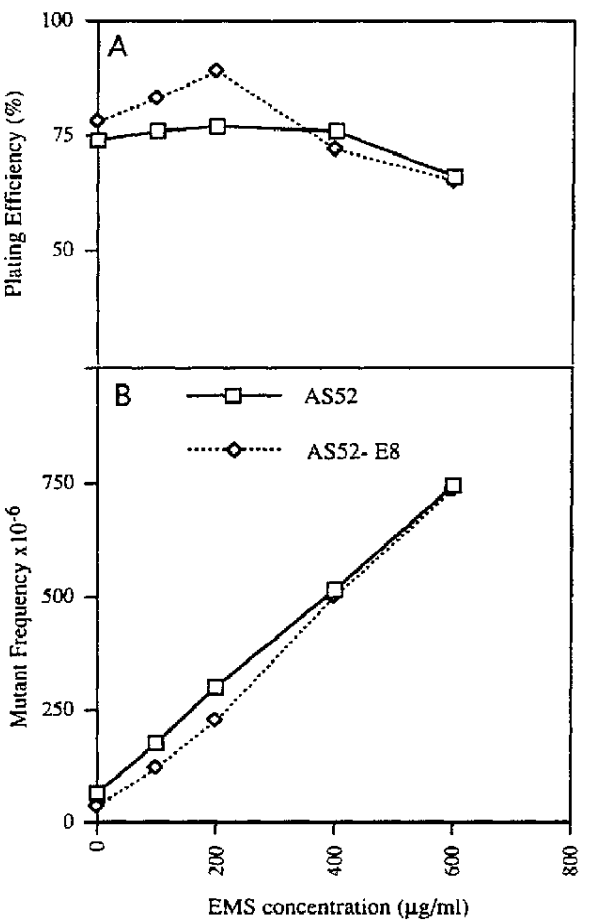

Fig. 1. Dose-dependent cytotoxic (A) or mutagenic (B) effects of the direct-acting mutagen EMS on AS52 cells and AS52-E8 cells expressing cytochrome P450 2A6.

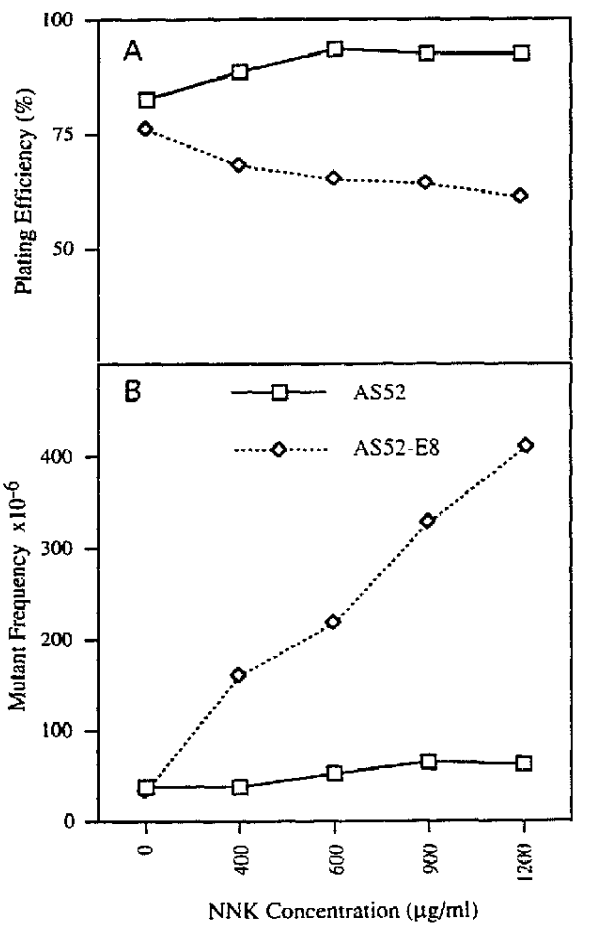

Fig. 2. Dose-dependent cytotoxic (A) or mutagenic (B) effects of NNK on AS52 cells and AS52-E8 cells expressing cytochrome P450 2A6.

Isolation of mutant clones for PCR and sequencing

For the isolation of mutants, 25 dishes were treated with 1200 μg/ml NNK for 24 h , two dishes were treated with 300 μg/ml EMS for 24 h as a positive control and two untreated dishes served as negative controls. After expression and selection, three 6-TG resistant clones were isolated from each of the two subcultures for each of the initial 25 NNK-treated dishes. This would ensure that at least 25, and up to 150, of the isolated mutants would be of independent origin. Dishes seeded to determine cloning efficiency and number of mutants indicated that the NNK mutation frequency was 339×10^{-6} and the background frequency was 24×10^{-6}, representing about a 14-fold increase over background for a 24 h NNK treatment, and agreeing with the increase in mutation frequency at this dose shown in Figure 2. The positve control (EMS, 24 h treatment) induced mutation frequency was 1435 mutants/10^6 cells in this experiment. Ninety-eight of 150 mutants isolated from NNK treated cultures survived the initial cloning and the dilution cloning. After expansion their DNA was isolated for PCR analysis and sequencing.

PCR amplification and analysis

The DNA isolated from each of 98 mutant (6-TG^r) clones was subjected to PCR using primers specific for the *gpt* gene and primers for exon 5 of the *dhfr* gene. The presence of the *dhfr* PCR product at 0.4 kb provides an internal positve control for each reaction. As shown in Figure 3, amplification of AS52 genomic DNA yields three bands. The 0.7 kb band is the PCR product for the *gpt* structural gene and the absence of this band (Figure 3, lane B) indicates a large deletion/rearrangement or the loss of a primer binding site in the structural *gpt* gene. The presence of this 0.7 kb band suggests a point mutation or small deletion in the *gpt* gene (Figure 3, lanes C–F). Lane A illustrates an event in which both the rearranged pSV2*gpt* insert (1.1 kb) and the *gpt* structural gene were deleted or rearranged, or lost a primer binding site. The complete absence of bands is expected in the negative control (lane G), and all PCR products are compared to a positive (AS52) control (lane H). This approach allows the rapid distinction of *gpt* deletions or rearrangements from putative point mutations.

The results of the primary PCR for the 98 mutant clones are presented in Table I. Of these, 77 (78.6%) had no alterations observable in the migration on an agarose gel of the 0.7 kb structural gene PCR product and are putative point mutants, while 21 (21.4%) show no band for the *gpt* structural gene and were attributed to deletions or rearrangements. The percentage of deletions/rearrangements after NNK treatment was quite different from those previously reported for the spontaneous mutants obtained from AS52 cells in which ~45% of mutations were attributed to large deletions/rearrangements (30). With a 14-fold enhancement of NNK-induced mutation frequency over background, 7.7% (1/13), or ~8/98 mutations may be of spontaneous origin, with approximately four (45% of eight) of these expected to be large deletions/rearrangements. Thus, NNK treatment induced ~17 of the 21 deletions/rearrangements found among the 98 mutants analyzed.

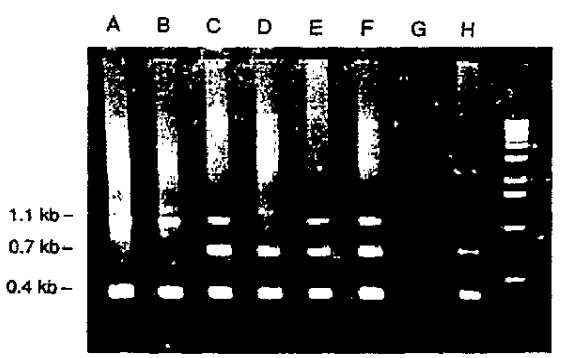

Fig. 3. Lane A, *dhfr* band (0.4 kb) is present, but both the *gpt* structural gene product (7 kb) and product of rearranged pSV2*gpt* insert (1.1 kb) are missing; lanes B and D, *dhfr* band and rearranged pSV2*gpt* band are present, but the PCR product for the structural gene is missing; lanes C, E, F, *dhfr*, *gpt* structural gene and rearranged pSV2*gpt* insert products are all present; lane G, negative control (no template DNA in PCR); 1 kb ladders are shown in left and right lanes.

Sequence analysis of putative point mutants

The bands containing the PCR product of the structural *gpt* gene (0.7 kb, Figure 3) were excised from gels and again PCR amplified as described in Materials and methods to generate the *gpt* gene fragment for seqencing. A summary of the sequencing data from the 77 mutant AS52-E8 clones for which PCR data indicated potential *gpt* point mutations is presented in Table II. Among these 77 mutants, 89% (68/77) resulted from base substitutions with 84% (64/77) transitions and only 5% transversions. The previously reported three-base deletion hotspot (30) accounted for only 8% of the total (Table II).

Table I. Primary PCR results for AS52-E8 mutants

	No. of mutants	%
No observable alterations[a]	77	78.6
Deletions/rearrangements[b]	21	21.4
Total of mutants analyzed[c]	98	

[a]No alterations were observable in the migration of the primary PCR products of the structural *gpt* gene on an agarose gel as compared to the AS52 wild-type control.
[b]Deletions or rearrangements resulting in the absence of PCR amplified *gpt* sequences.
[c]Due to the method used for mutant clone isolation, nine of these mutants are not necessarily of independent origin.

Table II. DNA sequence analysis of the *gpt* structural gene of 6TG^r-AS52/CYP2A6 clones following NNK exposure

	Number observed[a]	% of total complete *gpt* sequences
Sequences completed	77	100
Transitions	64	84
GC → AT	62	81
AT → GC	2	3
Transversions	4	5
GC → CG	1	1
GC → TA	3	4
AT → CG	0	
AT → TA	0	
Deletions	7	9
−6 base	1	1
−3 base	6	8
Complex	0	
No mutations observed in the *gpt* structural gene	2	3

[a]Mutants include 9/77 (12%) that are not necessarily of independent origin.

These data are strikingly different from those for spontaneous AS52 mutants in which 56% of the point/small deletion mutations occurred at the deletion hotspot, while base substitutions comprised only 24% of the total mutations and for which the majority (18%) were transversions (30). Two of the 77 mutants (Table II) produced no detectable change in the *gpt* coding sequence; however, any factors that ultimately affect the production or function of the gene product may lead to 6-TG resistance; thus, mutations or other changes that affect the promoter region may survive the selection process, but will not generate a detectable change in the sequence.

Figure 4 presents the spectrum of mutations induced in the *gpt* coding sequence of AS52-E8 cells expressing CYP2A6 following treatment with NNK. Of the 77 point mutants studied, only nine may be considered to be of non-independent origin (i.e. sibs) as they contained a mutation identical to that observed in another mutant arising from the same initial treatment dish. These possibly sib-selected clones are identified in Figure 4. Of the total of 68 base substitutions (including possible sibs), 64 are transitions and only four are transversions (Table II). The majority (62) of the transitions are GC to AT with 54 occurring at a guanine following a purine (RG site). About 70% of the GC to AT transitions at RG sites are on the non-transcribed strand. Six hotspots for base substitution, defined here as sites at which >5% of all point mutations occur, may be seen at base numbers 23, 27, 91, 116, 281 and 332. All of these involve GC to AT transitions at RG sites. Most of these hotspots (4/6 sites) accounting for 31% (24/77) of the point mutations occur at the same sequence motif (GGT) as is mutated in codon 12 of Ki-*ras* in strain A mouse lung tumors induced by NNK (21,22).

Discussion

The primary goal of the present study was to determine the types of mutational damage induced by the potential human carcinogen NNK after activation by an individual human cytochrome P450 (CYP2A6) known to metabolize this compound. For these studies, the CYP2A6 was transfected using a retroviral vector and expressed from the viral LTR promoter (28) in a mammalian cell (AS52) carrying a suitable target gene. The AS52 cell line was chosen because it is possible to obtain a direct sequence of the entire target gene (30) and because, as a derivative of the CHO cell line, it expresses negligible cytochrome P450 monooxygenase activity (37,38). That the AS52 parental cell line does not activate NNK is confirmed by the lack of cytotoxicity in these cells following treatment with NNK. The major outcomes of the study were that in the Chinese hamster ovary AS52 cells, human cytochrome 2A6 activation of NNK resulted in a significant increase in the number of GC to AT transitions in the *gpt* gene and a significant increase in the number of large deletions/rearrangements at this locus.

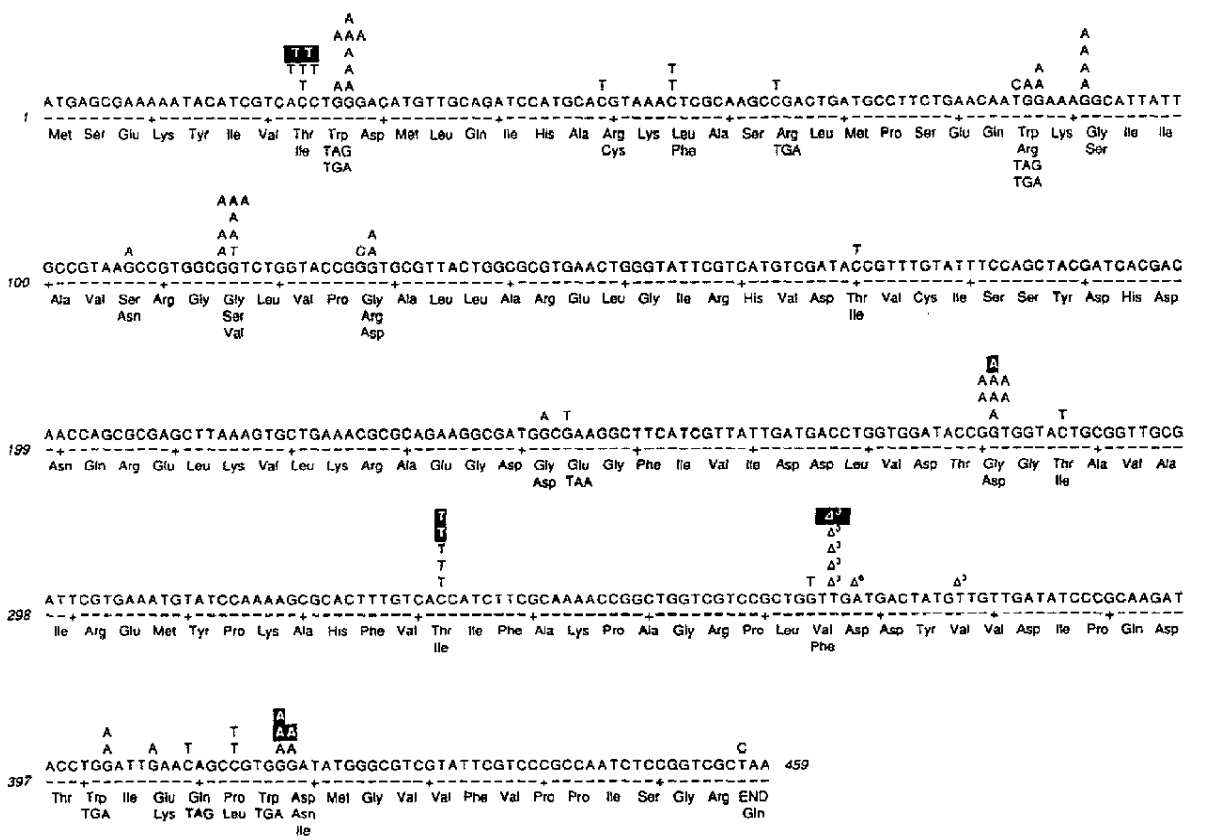

Fig. 4. Boxed base or symbol represents a possible sib-selected clone. These mutations are not necessarily of independent origin. Δ^1 represents a deletion of three bases. Symbol is placed at the first possible sequence deleted. Superscript number indicates the number of deleted bases in the mutation. In several of the deletions observed there exist multiple possible adjacent deletions that would yield the same primary mutant sequence.

The study of NNK is of basic interest because it can be metabolized through different pathways: two that lead to DNA damage are methyl hydroxylation, which yields a pyridyloxobutylating agent, and methylene hydroxylation, which generates a methylating intermediate (5–8). These reactive intermediates form DNA adducts that ultimately would generate differing mutational spectra. However, little is known about the types of mutational damage resulting from pyridyloxobutylation, although it is known to be mutagenic *in vivo* and *in vitro* (22,39). The most striking features of the spectrum shown in Figure 4 are the numbers and locations of the GC to AT transitions. The majority occur at a guanine following a purine (RG sites) with ~70% of these on the non-transcribed strand. There are several hotspots for base substitution; these are all at RG sites and result in GC to AT transitions, again primarily of the non-transcribed strand. Most of the GC to AT transition hotspots (4/6 sites with 24/77 mutations) occur at the second G of the GGT sequence motif. Interestingly, this is the motif of codon 12 in the Ki-*ras*. In lung tumors of strain A mice, a GC to AT transition in the second base pair of codon 12 was found to be the predominate mutation caused by NNK in the Ki-*ras* gene (21,40).

Previous studies to determine the mutational specificity of NNK have been conducted with bacterial systems. NNK activated by Aroclor-induced rat liver S-9 was found to be mutagenic in *Salmonella typhimurium* tester strains TA100 and TA1535, reflecting base substitution at GC base pairs (41,42). Mutational spectra of NNK obtained from the *lacI* gene of bacteria showed that GC to AT transitions accounted for 55–95% of the events depending on whether induced rat liver S-9 or host-mediated activation was used (43,44). In contrast to our data, these studies did not find the GGT motif to be a hotspot. It should be noted that a statistical analysis of the mutagenic spectra of various alkylating agents has demonstrated that these spectra can be strongly influenced by whether they were generated in a bacterial or in a mammalian system (45). In addition, Foiles *et al.* (39) have reported that AMMN (DNA methylating) was a more potent mutagen in the transfected *gpt* gene of V79 cells, while NNKOAc (DNA pyridyloxobutylating) was more potent in *Salmonella*. Thus, it is not yet clear whether the differences in the reported spectra are due to the use of bacteria, to the structure of the *lacI* gene as compared to the *gpt* gene, or to the possibility of multiple P450s being involved in these systems.

The mutation of codon 12 in *ras* has generally been attributed to the methylation of guanine to form O^6-methylguanine followed by mispairing. In our current study, GC to AT transitions were also the major event observed when NNK was activated by human CYP2A6 (Table II), also suggesting guanine O^6-methylation. However, a previous study of human CYP2A6, indicated that both methylene and methyl hydroxylation could occur (18). Thus, the spectrum should have reflected the formation of both methyl and pyridyloxobutyl adducts and should have included GC to TA transversions (22). Only 4/77 mutations of this type were seen (Table II), and these could be due in part to the spontaneous background. The reason for an apparent lack of a contribution by the pyridyloxobutyl adduct is unclear. Ronai *et al.* (22) have recently demonstrated that the *ras* gene in 47% of lung tumors from strain A mice which had been induced by a direct-acting pyridyloxobutylating agent (NNKOAc) also had GC to AT transitions at codon 12, while 53% had GC to TA transversions. Thus, pyridyloxobutyl adducts could have contributed to some of the GC to AT transitions we observed, but, if pyridyloxobutylation did occur, the low level of GC to TA transversions (Table II) remains unresolved. Furthermore, the data in Table I indicate that NNK activated by CYP2A6 did increase the number of deletions/rearrangements as expected for a bulky adduct, and pyridyloxobutylation could have contributed to this event. Also, the pyridyloxobutylating and methylating intermediates have different rates of DNA reactivity, so the relative contribution of the two α-hydroxylation pathways do not necessarily reflect the amount of each type of DNA adduct that would result (46). Another factor contributing to the high level of GC to AT transitions in our data is the deficiency in methyl transferase (47) in the Chinese hamster ovary derived AS52 cells which may bias the spectrum towards methylation damage. The strand bias of NNK intermediates for GC to AT transitions in the non-transcribed strand (Figure 4) is not without precedence for alkylating agents (48–51). It has been discussed whether differential repair between the two strands due to transcriptional activity accounts for the mutational preference (45,52,53). In our system repair by methyltransferase should not be a factor; thus, the relative distribution of mutable RG sites on each of the strands, or the structure of the *gpt* protein itself (54), may be a more significant factor in leading to the observed bias.

The cytochrome P450 used in this study, CYP2A6, has been demonstrated to be involved in human liver activation of NNK (20) and other members of the CYP 2A family have been demonstrated to be involved in NNK metabolism in various rodent tissues, including lung, a major target for NNK carcinogenesis (21,55–57). It is not clear which P450s may be involved in the activation of NNK in human lung (18). To date, CYP2A6 has not been detected in human lung, though it is possible that it may be expressed in only a small population of target cells and thus may be difficult to detect. Since rodent lung is known to be a major target for NNK carcinogenesis (1,2,40,57,58), identifying the P450s active in human lung tissues should be useful in identifying humans at greater risk of cancer due to tobacco use. An important question is whether some individual P450s may favor (or exclusively use) a methylating or pyridyloxobutylating pathway. Furthermore, although adducts of both intermediates appear to be involved, the relative contribution of the two pathways in the mutation/carcinogenesis processes requires further study. Knowing the specific P450s involved and the DNA-damaging effects of each intermediate will increase our basic understanding of the mechanisms by which NNK manifests its species/tissue-specific carcinogenic effects.

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